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Interlaboratory Variation in Oxygen Tension Measurement by Eppendorf ''Histograph'' and Comparison With Hypoxic Marker

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Background and Objectives: The median of pO_2 values in tumor measured by Eppendorf ''Histograph'' with a needle-type electrode has been used as a prognostic indicator in cancer patients. However, it is not established that a pretreatment measured pO_2 value can be used as a universal predictor of local control probability, because the variation in pO_2 values, especially in hypoxic tissue, among institutes may not allow comparison of measured ''absolute pO_2 values.'' The purpose of this study was to examine the variation in oxygen tension measurement by Eppendorf ''Histograph'' among six laboratories using a single batch of mice and tumors and the same detailed protocol. These results were also compared to the immunohistochemical staining of 2-nitroimidazole adducts.

Methods: C3H mice bearing FSaII murine fibrosarcoma subcutaneously were shipped to all laboratories, and the oxygen status in tumors and in normal subcutis was examined using Eppendorf ''Histograph'' and immunohistochemical hypoxic marker.

Results: All laboratories showed that the FSaII tumor was hypoxic with at least 77% of measured points under 10 mmHg in pO_2 and with a median pO_2 value less than that of normal subcutis. These results were further confirmed immunohistochemically. These findings are interpreted as evidence that the pO_2 values measured by Eppendorf ''Histograph'' can be useful. However, the median values of tumor pO_2 varied from 1.5 mmHg

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to 5.6 mmHg among the laboratories, and pO_2 of normal subcutis also varied from 28 mmHg to 38 mmHg. There were also significant differences in hypoxic fraction, defined as the fraction under a given oxygen partial pressure (i.e., under 2.5, 5, or 10 mmHg), among institutes.
Conclusions: Caution needs to be exercised in using the absolute, median, or distribution of pO_2 values measured by the Eppendorf ''Histograph'' to compare the data between laboratories or to predict the radiation response in an individual subject. *J. Surg. Oncol. 1997;66:30–38.*

KEY WORDS: interlaboratory variation; oxygen tension; mouse tumor; Eppendorf Histograph

INTRODUCTION

Oxygen tension in tumors has been suggested as an important determinant of response of cells and of tissue to radiation therapy and chemotherapeutic agents. Although there are several methods to evaluate the oxygen status in tumor, the polarographic oxygen electrode method is the only direct measurement of pO_2 in tissue [1]. The pioneering work of Kolstad [2] using a Clark-type electrode indicated that the cervical cancer patients with pO_2 values <10 mmHg had higher recurrence rates. Gatenby et al. [3], also using the polarographic method, reported that head and neck cancer patients with >26% of their tumor volume with pO_2s less than or equal to 8 mmHg showed a poorer radiation response than patients with high pO_2 readings. Among several devices available for the polarographic measurement of oxygen tension, the Eppendorf ''Histograph'' (Eppendorf, Hamburg, Germany) has been used most widely, not only in animal studies [4] but also in human clinical studies, because of its stability, short sampling time, ease of use, and computerized control system. The correlation between the clinical outcome and the pO_2 values measured by the Eppendorf ''Histograph'' was first reported by Höckel et al. [5], for patients with cancer of the uterine cervix. Those patients whose tumor showed a median pO_2 value <10 mmHg had significantly poorer prognosis. They recently reported another prospective study that identified tumor oxygenation and International Federation of Gynecology & Obstetrics (FIGO) stage as the most important independent prognostic factors [6]. In addition, tumor oxygen tension in breast cancer patients measured by the Eppendorf ''Histograph'' at several institutes was reported to be an important modifier of the radiation dose response [7]. These studies have demonstrated the value of tumor pO_2 as a prognostic factor for radiation response.

In a laboratory, the stability, reproducibility, and compatibility with other mehtods of this Eppendorf system have been established [8–10]. No one knows, however, how large the interlaboratory variation is. In other words, it is not established that a fixed pretreatment median pO_2, e.g., 8 or 10 mmHg, can be used as a universal predictor of local control probability because the variation in pO_2 values among institutes or instruments may not allow the comparison of absolute pO_2 values. To address this problem, we investigated the interlaboratory variation in pO_2 values measured by Eppendorf ''Histograph'' in six laboratories using a common biological system. We also compared these results to the immunohistochemical staining of adducts of EF5, a 2-nitroimidazole [11]. This study would indicate whether the median value or distribution of pO_2 measured by the Eppendorf ''Histograph'' can serve as a prognostic factor in worldwide use.

MATERIALS AND METHODS

Animal and Tumor

The spontaneous fibrosarcoma (FSaII) of the C_3H_f/Sed mouse was implanted into the subcutaneous space of the right hind leg of 7–8-week-old C3H male mice at Edwin L. Steele Laboratory in Massachusetts General Hospital (Boston). Ten mice were sent to each laboratory for pO_2 measurement by Eppendorf ''Histograph'': Dana-Farber Cancer Institute, Duke University Medical Center, M.D. Anderson Cancer Center, University of Minnesota, and Institut Gustave-Roussy (France). Mice were also sent to the University of Pennsylvania for characterization of hypoxic cells by immunohistochemical examination. Therefore, seven laboratories participated in this project. All laboratories had obtained approval from their institutional animal care committee. All laboratories except the University of Pennsylvania were randomly designated A–F.

Oxygen Tension Measurements by Eppendorf ''Histograph''

The common protocol used by six laboratories defined the experimental procedures in the greatest possible detail. The ''Histograph'' was calibrated for at least 15 min according to manufacturer's direction. It was set so that the steplength was 0.4 mm and the overstroke was 0.3 mm. This means that the needle-type electrode automatically moved 0.7 mm forward and then 0.3 mm backward every 1.4 sec, making a pO_2 determination at each step. The oxygen tension was measured when the tumor reached between 8 mm and 9 mm in diameter. The mice

were anesthetized by the mixture of Ketamine (100 mg/kg) and Xylazine (10 mg/kg), i.m. and kept on a heating pad to maintain body temperature between 37 and 38°C. The abdominal hair was shaved, and an electrocardiogram patch was attached as an anode. The oxygen partial pressure was measured by the Eppendorf ''Histograph'' 30 min after the onset of anesthesia. The pO_2 was measured in the tumors of at least 6 out of 10 mice and in the subcutis of the left hind foot dorsum in 2 out of the 10. Values of 50–60 were obtained from each tumor through four or five parallel tracks.

Immunohistochemical Analysis

Immunohistochemical staining using EF5, a pentafluorinated derivative of etanidazole [2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide], was done as reported previously [11]. Briefly, 3 hr after the injection of a 10 mM solution of EF5 via the tail vein (equivalent whole-body concentration of 100 μM), the tumors were removed from the anesthetized mice and cut roughly in half. One half was disaggregated in an enzyme cocktail and then the cells were fixed (4% buffered paraformaldehyde, 0°C, 60 min) and blocked to prevent nonspecific binding. The cells were stained with the anti-EF5 monoclonal antibody named ELK-51 conjugated with the red-fluorescent dye, Cy3, and analyzed by a flow cytometer. The other half of each tumor was quick-frozen and subsequently sectioned. Sections (14 μm thick) were fixed, blocked, and stained as with the cells. The sections were examined and photographed with a fluorescence microscope.

Data Analysis

A histogram of the pO_2 distributions, with a class width of 2.5 mmHg, was made from each tumor and the cumulative fraction curve was drawn by summing up the columns successively from 0 mmHg. From this curve, the median and the fraction under a given oxygen tension, such as 5 mmHg or 10 mmHg, were obtained. The mean and standard error of these fractions from each laboratory were calculated and compared to the results of other laboratories using the *t*-test. The median values among laboratories were compared using Kruskal-Wallis test. Additionally, all pO_2 values obtained from several tumors in one laboratory were grouped together and presented as a histogram. To examine the degree of the variation among the laboratories, the coefficient of variation of the median values among all laboratories was calculated and compared to the coefficient of variation calculated from all individual tumor data in each laboratory.

RESULTS

General Condition of Animals and Tumors

The animals were shipped by overnight flight to the laboratories in the United States and France. The mice arrived in 24 hr after shipment, except at laboratories B, F, and the University of Pennsylvania, where the mice were received between 24 and 56 hr after shipment. The condition of the animals was good except at laboratory F; one mouse died before the experiment and another died during anesthesia. The weight of the mice in laboratory F varied from 18 g to 32 g, whereas in other laboratories, from 28 g to 32 g. Some of the mice in laboratory F seemed to suffer from dehydration during the shipment and did not recover. Therefore, the data from laboratory F were removed. The number of measured tumors varied from 6 to 9, mainly because the size of some tumors exceeded what was defined in the protocol. Histological examinations with the 2-nitroimidazole EF5 were done in five tumors with other tumors serving as controls.

Oxygen Partial Pressure in FSaII Tumors

Figure 1A shows the histograms of FSaII tumors from six laboratories. All distributions show the same tendency, i.e., the highest frequency of pO_2 in all laboratories was <7.5 mmHg. At least 77% of measurements were <10 mmHg (Fig. 1B). All laboratories showed that the FSaII tumor was hypoxic. However, there were some quantitative differences among laboratories. The median pO_2 values varied from 1.5 mmHg to 5.6 mmHg (data from laboratory F are not included in analysis). The distribution of pO_2 values was also different. Laboratory B showed a number of high pO_2 values, including >60 mmHg. Laboratories C and D showed very high frequency in the 0–2.5 mmHg range. Laboratories A and E showed a broader distribution. The shapes of the cumulative curves were also different from one another (Fig. 1B). The accumulated fraction <2.5 mmHg varied from 16% to 88%; those <5 mmHg ranged from 41% to 94%; and those <10 mmHg ranged from 77% to 98%. These results were statistically compared. Inter-laboratory variations in fractions <2.5 mmHg, 5 mmHg, and 10 mmHg were substantial and for most comparisons were significant by *t*-test (data not shown). The median values of each group showed significant difference by the Kruskal-Wallis test ($P < 0.0001$). Intertumor variations in median pO_2 values among tumors in the same laboratory and interlaboratory variations in median pO_2 values among different laboratories were quantified by the coefficient of variation (Table I). The interlaboratory variation was within the same range as the intertumor variation.

Oxygen Partial Pressure in Normal Subcutaneous Tissue of Left Hind Foot Dorsum

Figure 2A shows the pO_2 histograms of the normal subcutis. The distribution patterns of four laboratories (A, B, C, and D) were similar and had a ''normal'' distribution. Those of E and F were different and had some negative and quite low pO_2 values. The median pO_2 val-

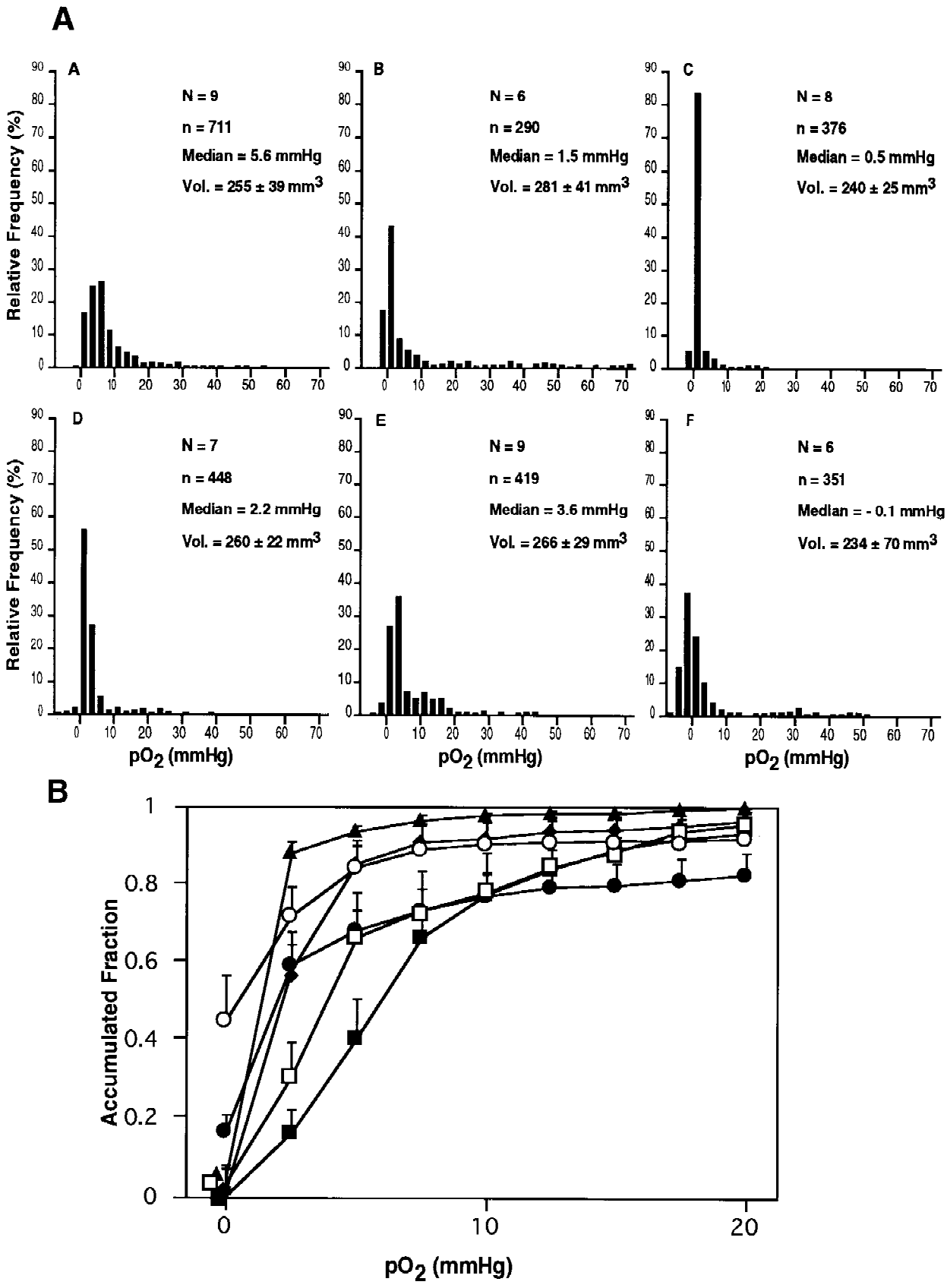

Fig. 1. **(A)** Histograms and **(B)** cumulative fraction curves of FSaII tumors. The animal condition of laboratory F was poor. N: the number of animals; n: the total number of measurements; Median: the median pO_2 value; Vol.: tumor volume. The column width is 2.5 mmHg. The cumulative curves were drawn by summing up the columns of the histograms of each tumor one by one from 0 mmHg and then calculate the mean and standard error at each point. Error bars represent the standard error. ■: Laboratory A, ●: B, ▲: C, ◆: D, □: E, ○ :F

TABLE I. Coefficient of Variation of Median pO_2 Values

Animal no.	Median pO_2 value of each tumor in each laboatory (mmHg)[a]						Median pO_2 value of each group (mmHg)
	A	B	C	D	E	F	
1	8.6	1.0	0.4	1.9	1.6	(2.6)	A:5.6
2	3.8	2.5	0.4	3.5	2.7	(1.1)	B:1.5
3	2.9	5.8	0.3	2.6	7.2	(0.1)	C:0.5
4	5.9	1.3	1.1	2.6	1.5	(−3.1)	D:2.2
5	6.9	1.6	0.3	3.2	2.7	(0.0)	E:3.6
6	8.0	0.4	0.3	1.3	4.6	(0.4)	
7	6.6		0.4	1.7	2.2		
8	3.5		0.9		13.7		
9	2.6				3.3		
Coefficient of Variation	0.42[b]	0.92	0.60	0.34	0.90		0.74[c]

[a]Median values obtained from all tumors presented here.
[b]Coefficient of variation of the median pO_2 values of each tumor.
[c]Coefficient of variation of the median pO_2 values of each institute except laboratory F.

ues in individual laboratories varied from 28 mmHg to 38 mmHg (the data from F not included). Even in the four laboratories that showed the same distribution pattern, the median varied from 29 mmHg to 38 mmHg. The different distribution observed by laboratories E and F is also evident in the cumulative curves, shown in Figure 2B. The slopes of four laboratories were similar, but the other two laboratories showed a flatter curve. In all laboratories, the pO_2 of normal subcutis was measured to be higher than for the tumor tissues.

Immunohistochemical Staining

Five FSaII tumors were examined by the immunohistochemical method. There was a good correlation in the degree and extent of binding between the flow cytometric and the histopathologic results. Four of five tumors exhibited bright staining (presumed hypoxia) using both analytic techniques. Flow cytometric analysis showed a median and maximum fluorescence in one experimental tumor of 62.6 and 400.0, respectively (Fig. 3A), whereas corresponding values for non-EF5-treated controls were 6.7 and 10.7. The histological section corresponding to the experimental tumor showed large regions of bright binding (Fig. 3A). In contrast, another experimental tumor showed 8.7 and 70 as a median and the highest intensity in cytometry data, and the histology section showed only dim binding with one small bright area (Fig. 3B). In addition, heterogeneity of binding was found within and between tumors. Variations (10-–20-fold) in binding within an individual tumor were found, and a sevenfold variation was observed between tumors, based on the median values (data not shown).

DISCUSSION

Many factors can affect the results of these pO_2 measurements in many laboratories. These factors can be classified into four categories: (1) materials (animal and tumor), (2) methods, (3) electrodes and machine itself, and (4) human factors. The purpose of this study was to determine the variation of pO_2 values measured by the Eppendorf ''Histograph'' electrode system in several laboratories by reducing as much as possible the variability of factors. To this end, we prepared all animals and cells from the same laboratory and tried to maintain the same experimental condition (such as body temperature and anesthesia). We also defined the movement of the needle-type electrode, the calibration procedure, and the anode. Therefore, the main factors that may have affected the results in this study were the factors of the machine and/or the needle electrodes themselves and the human factor. Therefore, the results of this study should estimate the minimum variation among institutes. In other words, it is generally assumed that those factors are negligible as long as the device is operated according to instructions. The present study for interlaboratory variation was designed to test this assumption.

All laboratories showed that FSaII tumors were hypoxic compared to normal subcutis, which is consistent with other published data [12]. Moreover, these results were consistent with the immunohistochemical analysis. Both methods showed that most of these tumors were hypoxic and also detected remarkable heterogeneity within and between tumors. Using the Eppendorf machine, intertumor variation in the same laboratory and interlaboratory variations were in the same range, and the median pO_2 values varied by a factor of 3–10 (Table I). Flow cytometry data also showed that the median fluorescent intensity varied by a factor of 7 among examined tumors (data not shown). These facts indicate that the pO_2 values measured by Eppendorf ''Histograph'' can provide semiquantitative estimates of tissue pO_2.

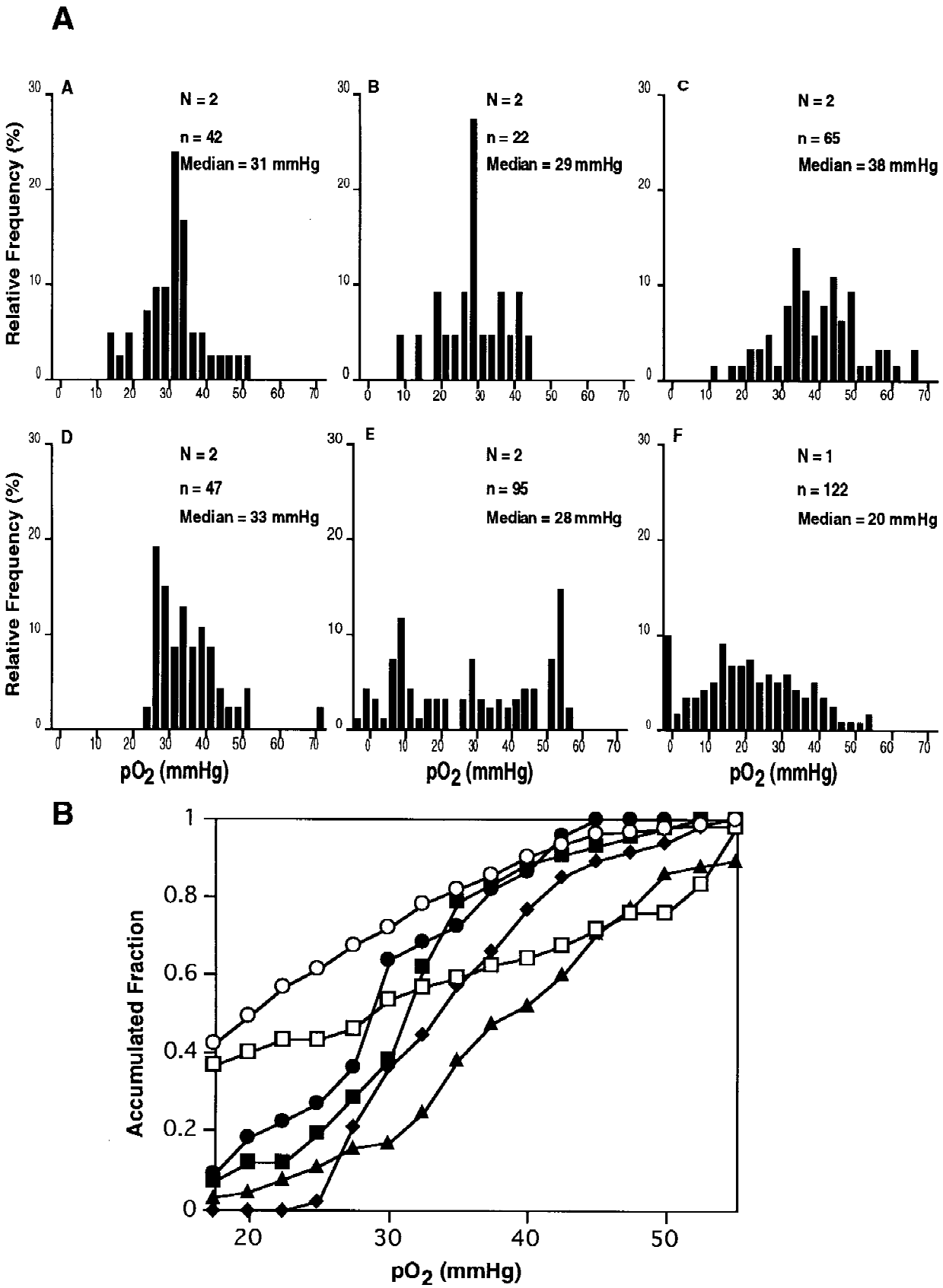

Fig. 2. **(A)** Histograms and **(B)** cumulative fraction curves of the normal subcutis of the left hind foot dorsum. The animal condition of Laboratory F was poor. N: the number of animals; n: the total number of measurement; Median: the median pO_2 value. The cumulative curves were drawn by summing up the columns of the histograms one by one from 0 mmHg and then was shown from 17.5 to 55 mmHg.■: Laboratory A, ●: B, ▲: C, ◆: D, □: E, ○ :F

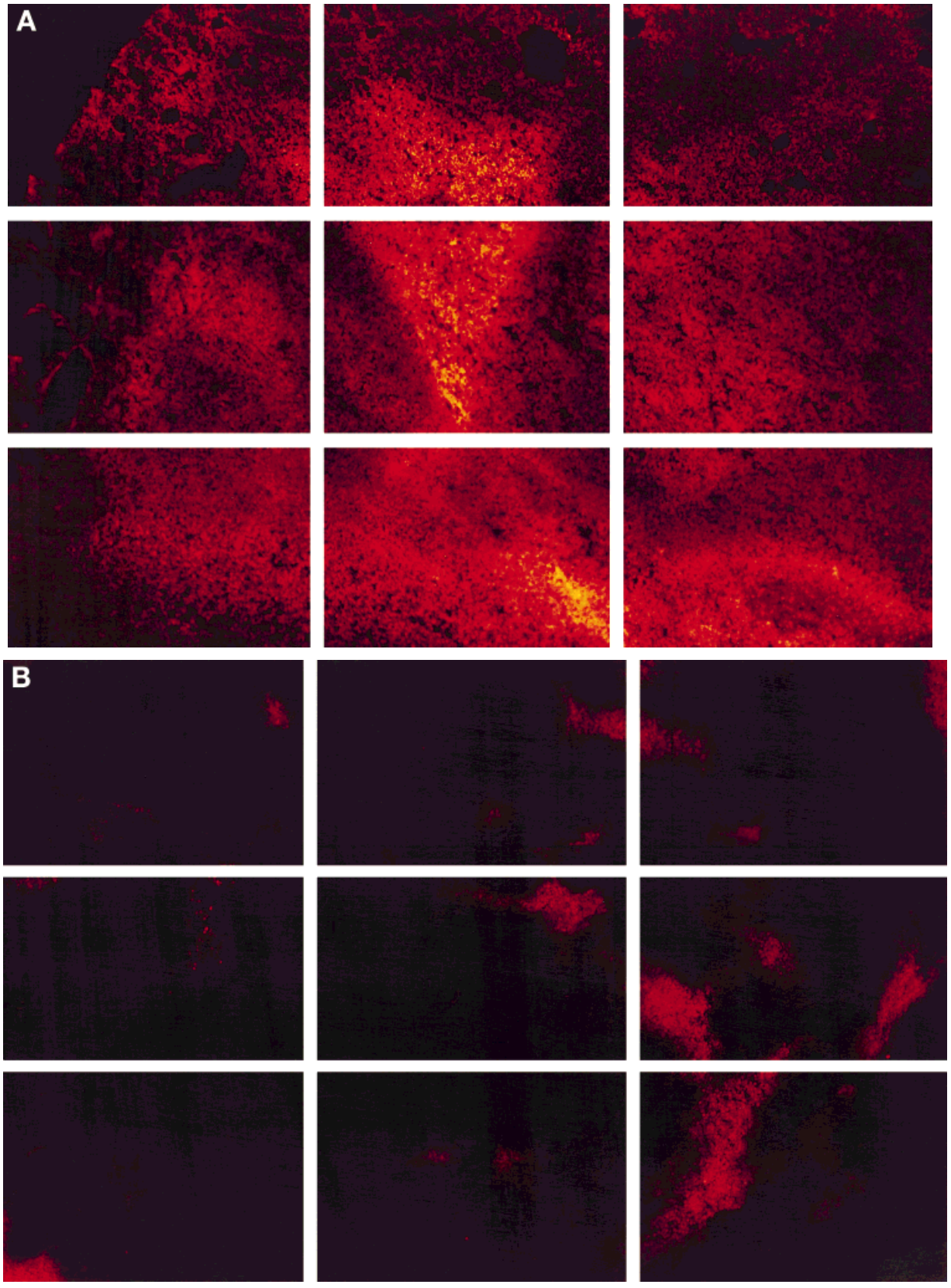

Fig. 3. Immunohistochemical staining of FSaII tumors. These photographs covered about one-fourth of the tumor cut surface. The intensity of the red fluorescence is inversely proportional to the oxygen tension. Each of the nine adjacent fields is 1.2 mm × 0.8 mm. **A.** One of the hypoxic tumors. Most of the presented area showed extensive bright binding, suggesting low pO_2 levels. **B.** Well-oxygenated tumor. Most fields showed a dim appearance of fluorescence. Some small bright areas could be detected. The exposure (camera shutter) for the second panel was 2.5× longer than the first, indicating that the true difference in binding is considerably greater than what the photos show. Tumor sections from non-EF5-treated animals were uniformly dark at these exposure levels (data not shown).

However, if absolute pO_2 measurements are to be used as prognostic parameters, these differences were considerably larger among the different institutes. The fact that the median values varied from 0.5 mmHg to 5.6 mmHg suggests that to use the absolute median pO_2 value like 10 mmHg as the criterion for hypoxia [5, 6] may not be applicable for all institutes. The large difference in fractions of measurements <7.5 mmHg among institutes implies that to apply 26% or more of the fraction <8 mmHg as a threshold [3] could not be reproducible in other laboratories.

Negative pO_2 values were another problem observed in all laboratories, especially in laboratories D, E, and F where pO_2 values under −2.5 mmHg were obtained. Reasons for negative pO_2 values were not clear. The experimental data of Eppendorf suggest that the standard deviation of the measurements by this set up should be ~1 mmHg (pers. comm. with Dr. Slupek, Eppendorf, Germany, November 1994). This mean that if the true pO_2 value of the tissue is 0 mmHg, the 95% of measured pO_2 value can vary between-2 mmHg and 2 mmHg (i.e, 2 S.D.). If >5% of values are beyond this range of variation, the negative values can be caused by artifacts such as an excessive pressure change on a needle tip or electrical field changes. Poor condition of the electrode, the machine itself, or connection of the anode to the animals can also cause negative pO_2 values in measurements. In laboratory F, the condition of the animals was not satisfactory, and this coincided with many negative pO_2 measurements (Fig. 1). For the fourth animal in laboratory F (Table I), all values were under-2 mmHg. This suggested that other problems occurred during the measurement. Although this result could be dropped in a usual study, we decided to present all data obtained in this study. These data could show all problems observed in the pO_2 measurements using this setup.

These murine tumor data may not be directly applicable to human tumors, because human tumors are usually better oxygenated than the murine tumors [12]. This issue is partly addressed by the data of the normal subcutis. There was a significant variation even in the data of the normal subcutis. The median pO_2 values varied over 9 mmHg. This fact indicates that the absolute pO_2 values obtained by the Eppendorf ''Histograph'' can vary among insitutes, not only in murine tumor and subcutis but possibly also in human tumors.

Large variation in pO_2 measurements suggested that it is potentially beneficial to use more than one assay of pO_2. Our data suggest that EF5 binding study may provide valuable information on the distribution of hypoxia (level and extent). Although in the present experiment, EF5 binding study and pO_2 measurement by Eppendorf ''Histograph'' were performed in different tumors, it would certainly be possible to use needle electrode measurements and EF5 binding in the same animal. The variations in binding of EF5 seen within and between individual tumors of equivalent source suggest that some of the variations seen with the Eppendorf ''Histograph'' may be statistical in nature.

Conclusions

Caution should be exercised in comparing the measured ''absolute'' pO_2 values by the Eppendorf ''Histograph'' among institutes or applying these data to predict the radiation response at this stage of study. However, this conclusion does not deny the reliability of the Eppendorf ''Histograph'' as a semiquantitative instrument for oxygen measurement. At least, all laboratories showed that FSaII tumors were more hypoxic than the normal subcutis. Moreover, in the same laboratory, the pO_2 distribution of some murine tumors was reproducible [8]. As the next step, we should determine which factors can most affect the measurement of pO_2. Through this process, we can reduce the variability of the pO_2 values among laboratories and hopefully obtain a true pO_2 standard.

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